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Site-specific modification of the lactose operator with acetylaminofluorene

Gerhard Stöhrer*, Judith A.Osband* and Gabriel Alvarado-Urbina+

*Department of Chemical Carcinogenesis, Sloan-Kettering Institute for Cancer Research, Rye, NY 10580, USA and +BioLogicals, Ottawa, K1Y 4P1 Canada

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ABSTRACT

We have synthesized the tetradecamer GAGCXGATAACAAG containing a part of the sequence of the lactose operator. A guanine base in the sequence is replaced by the adduct of the carcinogen 2-acetylaminofluorene with guanine. Under the standard conditions of de-protection, the fluorene moiety is lost, leaving behind a guanine oxidation product. New conditions of de-protection have been developed which allow the isolation of an oligonucleotide containing the adduct of 2-aminofluorene with guanine. The presence of the aminofluorene adduct greatly increases retention on reverse phase chromatography and produces a unique pattern of sequencing bands.

INTRODUCTION

A critical step in chemical carcinogenesis is the covalent interaction of active carcinogens with DNA (1, 2). Proximate carcinogens are electrophiles which react with DNA to form covalent adducts, cross-links within DNA and with proteins (3) and apurinic sites (4) as the so-far identified lesions. There is little doubt that this spectrum of DNA lesions is responsible for the mutations and carcinogenic transformations induced by chemical carcinogens, but in no case has any one lesion been linked unequivocally to a biological effect since identity and position of any lesion have been only statistically known.

We have taken advantage of the newly-developed solid-state oligonucleotide chemistry to prepare an oligonucleotide with a bulky carcinogen adduct in a defined position. The carcinogen of choice was acetylaminofluorene, one of the most extensively studied chemical carcinogens (1). In its ultimate reactive form this carcinogen leads to four major adducts with guanine bases in DNA. Treatment of DNA *in vitro* with N-acetoxy-N-acetyl-2-aminofluorene (N-acetoxy-AAF) produces two adducts to guanine, the major one being N-(guanin-8-yl)-AAF, with 3-(guanin-N^2-yl)-AAF formed to a lesser degree (5).

A third adduct is unstable and leads to depurination of DNA (4). The two

stable adducts are also found in rat liver after treatment with AAF or N-hydroxyl-AAF (6), but the major DNA-bound AAF metabolite is a N-(guanin-8-yl)-2-aminofluorene (AF) derivative (7). The AF derivative, formed after activation by N,O-acyl-transfer (8), differs from the C-8 AAF derivative in the way the fluorene moiety is oriented relative to the double helix. Studies with DNA containing the AAF adduct indicate a disruption of the normal anti conformation of the adduct nucleoside with consequent severe helix distortion (9, 10), while the AF adduct can exist in the normal anti conformation with the AF moiety in the major groove of the helix (11, 12). Recently, it has been shown that different repair systems in E. coli act on the two types of adducts (13).

Here we describe the synthesis of a 14-mer oligonucleotide, site specifically modified with the bulky base adduct guanin-8-yl AF. A tetramer containing 6-O-methylguanine has recently been described (25).

MATERIALS AND METHODS

One-Step Preparation of N-Acetoxy-acetylaminofluorene

The procedure of Cramer et al. (14) was modified as follows: 2-nitrofluorene, 10 g 47.5 mmol, was dissolved in 250 ml hot ethylacetate and filtered. To the solution at ~35° was added 40 ml triethylamine, 35 ml acetic anhydride and 1 g Pt/C. The solution was hydrogenated at 35 psi pressure for 20 min when no further hydrogen was consumed. The catalyst was filtered and the solution carefully extracted with 1 M sodium bicarbonate. The organic phase was dried and evaporated yielding a crude product which consisted of 90% N-acetoxy-acetylaminofluorene and 10% acetylaminofluorene.

2'-Deoxyguanosin-8-yl-AAF (1)

The procedure of Cramer et al. (14) was used as far as the isolation of the crude adduct. This was chromatographed in two portions on a single Waters Prep 500 ODS cartridge. Half of the crude adduct from a 10 g N-acetoxy-AAF + 10 g deoxyguanosine incubation was pumped onto the column in 500 ml 50% methanol/0.05 M triethylamine acetate, pH 7.0. This eluted unreacted deoxyguanosine. The purified adduct was eluted with 80% methanol/0.05 M triethylamine acetate, pH 7.0, and crystallized after evaporation. The yield was 3.0 g or 6.25 mmol.

(2-N-Isobutyryl-2'-deoxyguanosin)-8-yl-AAF (2)

The procedure of Ti et al. (15) using transient protection by trimethylchlorosilane was used but the desilylation was done with saturated aqueous $NaHCO_3$ for 6 hr instead of aqueous ammonia and the product isolated after

flash chromatography on SiO_2. The yield was 2.5 mmoles of a yellow glass.

(5'-O-Di-p-anisylphenylmethyl-2-N-isobutyryl-2'-deoxyguanosin)-8-yl-AAF (3)

Dimethyoxytritylation was done according to Buchi (16). Flash chromatography on SiO_2 eluted with MeOH/TEA/$CHCl_3$ 2:20:78 yielded 2.2 mmoles of crystalline 5' DMT,N-ibu adduct $\varepsilon_{300} = 25.6 \times 10^3$ in ethanol. 100 MHz NMR spectrum in DMSO-d_6:isobutyl-methyl doublet 1.09 and 1.15 PPM; N-acetyl 2.12 PPM; DMT-methyl doublet 3.68 and 3.82 PPM; 5' H multiplet 4.6 PPM.

(5'-O-Di-p-anisylphenylmethyl-2-N-isobutyryl-3'-chloro-methoxyphosphityl-2'-deoxyguanosin)-8-yl AAF (4)

To 50 µl of CH_3OPCl_2 in 3 ml dioxane containing 2.6-lutidine was added dropwise 3, dissolved in 1.5 ml dioxane. The resulting phosphitylated nucleoside was dissolved in pyridine and used for oligonucleotide synthesis.

Oligonucleotide Synthesis and Purification

The automated solid-state DNA synthesizer of BioLogicals was used (17) and the modified base added through the spare port. Methyl groups were removed from the phosphotriesters by triethylammonium thiophenoxide in dioxane (18). For removal from the support and removal of base protecting groups, 10 mg of solid support was incubated with 1 ml 28% aqueous ammonia containing 10 µl mercaptoethanol under nitrogen for 4 hr at 50°.

The supernatant was lyophilized and desalted on a 25 ml Sephadex G25 column equilibrated with 0.1 M TEA bicarbonate, pH 8.7. The high molecular fraction was then loaded on a 2.5 ml DEAE cellulose column and eluted with a 100 ml gradient 0.25 M - 2 M TEA bicarbonate. Material eluting at the peak and beyond was pooled, lyophilized and loaded on an analytical ODS-3 reverse phase column, eluted with a 200 ml gradient of 10% to 30% acetonitrile in 0.1 M TEA acetate, pH 7.0. With this elution gradient the adduct-containing oligonucleotide is retained more strongly by the reverse phase column compared with an oligonucleotide lacking the lipophilic fluorene moiety.

Sequence Analysis

Oligonucletodies were end-labeled with polynucleotide kinase and the sequence analyzed using base-specific reactions (19, 20). Electrophoresis gels were 60 cm long and contained 24% acrylamide (21).

Oxidative Decomposition of Deoxyguanosin-8-yl-AF

Deoxyguanosin-8-yl-AAF, 10 mg, dissolved in 0.5 ml 28% ammonium hydroxide was sealed in a glass tube under air and heated at 60° for 21 hr. After lyophilization, the products were separated on an analytical reverse phase ODS-3 HPLC column, eluted with a 200 ml gradient of 15-80% acetonitrile in 0.1 M ammonium formate, pH 4.7.

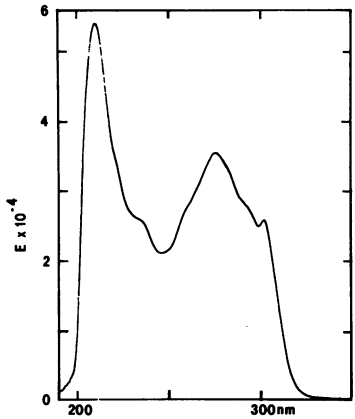

FIG. 1. Ultraviolet absorption spectrum of (5'-O-di-p-anisylphenyl-methyl-2-N-isobutyryl-2'-deoxyguanosin)-8-yl AAF in ethanol.

RESULTS AND DISCUSSION

Synthesis of 2'-Deoxyguanosin-8-yl-AAF-activated Nucleotide

If a modified base such as a base-adduct with a carcinogen is to be incorporated into an oligonucleotide, three conditions have to be met: the modified deoxynucleoside must be available in millimolar quantities, reactive substituent groups must be protected and the whole molecule must be stable towards the reagents used in the synthesis cycle and after synthesis must be stable during deblocking. Deoxyguanosin-8-yl-AAF satisfied the first two conditions very well. The third was satisfied with new reaction conditions described here.

The N-acetyl is hydrolyzed partially by the standard butyrylation conditions (16), but is stable under the milder conditions of transient silylation (15), particularly if desilylation is done with sodium bicarbonate. After subsequent tritylation, the nucleoside contains the intact AAF group as indicated by the UV-spectrum (Fig. 1) and the NMR-spectrum (not shown, key resonances are given in MATERIALS).

Assembly of the Modified Tetradecamer

The sequence chosen is part of the 17-mer minimal length "upper" strand of the lactose operator (22) containing a 3' G as part of a linker sequence:

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     5' G A G C X G A T A A C A A G
A A C A C T C G C C T A T T G T T  5'
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Shown here is the modified 14-mer as it would look after base pairing to the 17-mer minimal length "lower" strand. The synthesis of the oligonucleotide was conducted after a test with 0.1 mmole of phosphitylated deoxyguanosin-8-yl-AAF showed coupling in a yield close to that of phosphitylated deoxyguanosine (Fig. 2). The assembly of the modified 14-mer, with 0.4 mmol of 4

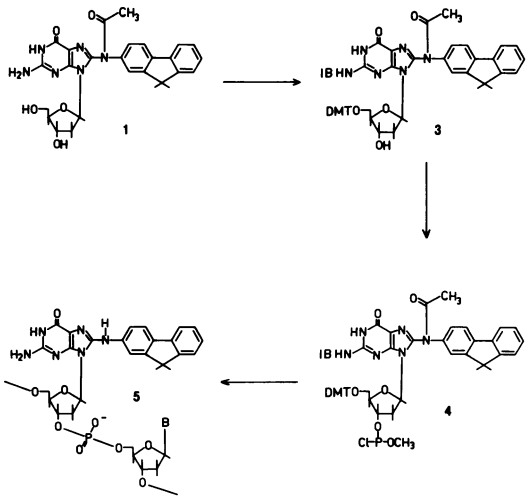

FIG. 2. Synthesis of modified oligonucleotide. Adjacent nucleotides in structure 5 are not shown.

added at the appropriate synthesis cycle through the spare port, resulted in an overall yield based on dimethoxytritanol of 72%.

Deblocking and Stability of Guanyl-8-AAF Linkage

For the basic conditions as used for the removal of N-acyl groups, the stability of deoxyguanosin-8-yl-AAF had to be reinvestigated. Kriek et al. (23) have described the conversion of guanosin-8-yl-AAF to guanosin-8-yl-AF after treatment with 0.1 N NaOH. Figure 3 shows the reaction of deoxyguanosin-8-yl-AAF with aqueous ammonia (curve 4) and aqueous sodium hydroxide (curves 1-3). Measured is the formation of deoxyguanosin-8-yl-AF at the characteristic absorption maximum of 325 nm (23). Curve 1 shows the reaction with 1 N NaOH at room temperature. The amount of deoxyguanosin-8-yl-AF goes through a maximum at 10 min and then decreases. The subsequent reaction which leads to the loss of absorption at 325 nm appears to be an air

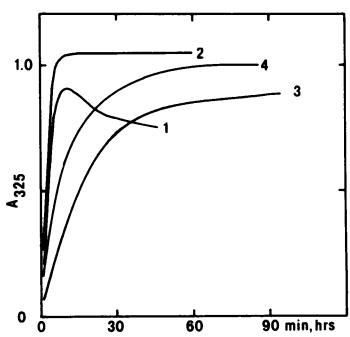

FIG. 3. Rate of hydrolysis of 2'-deoxyguanosin-8-yl AAF to 2'-deoxyguanosin-8-yl AF measured by the increase in A_{325}. Rates 1-3 are measured in minutes at 21° and are for the hydrolysis of 1 N NaOH (1); 1 N NaOH containing 0.3% 2-mercaptoethanol (2) and 0.1 N NaOH (3). Rate 4 is measured in hours and is for the hydrolysis in 28% aqueous ammonia containing 0.3% mercaptoethanol.

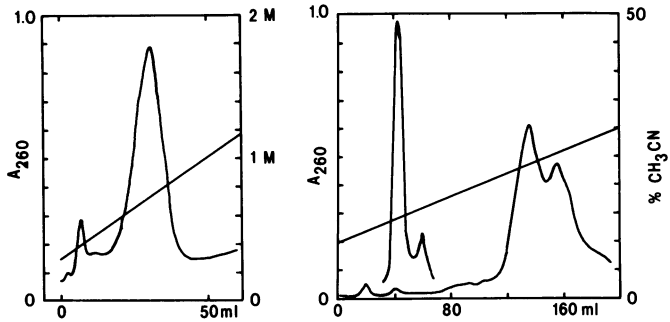

FIG. 4. Left panel. Elution diagram of deblocked AF-containing 14- mer reaction mixture on 2.5 ml DEAE cellulose eluted with a gradient of 50 ml 0.25 M TEAB to 50 ml 2 M TEAB.
Right panel. Elution diagram of oligonucleotides on an analytical ODS-3 reverse phase column eluted with a gradient of 10- 30% acetonitrile in 200 ml 0.1 M TEA-acetate, pH 7.0.

oxidation reaction, since it can be suppressed by the addition of mercaptoethanol. This is shown in curve 2 where the hydrolysis is done in 1 N NaOH containing 0.3% mercaptoethanol. Curve 4 is the hydrolysis in 28% aqueous ammonia containing 0.3% mercaptoethanol (with different time scale). At the level of oligonucleotide this oxidation is so substantial that our first isolation of oligonucleotides, using the standard hydrolysis conditions followed by thin-layer chromatography, produced a 14-mer that had entirely lost the fluorene moiety as judged by the absorption at 300 nm. Hydrolysis in the presence of mercaptoethanol suppresses the oxidation also at the oligonucleotide level, both with sodium hydroxide and ammonium hydroxide.

Products of the Oxidation of Deoxyguanosin-8-yl-AF

We treated deoxyguanosin-8-yl-AAF under oxygen with ammonium hydroxide under conditions slightly more severe than those used in the standard deblocking reaction. The products on reverse phase chromatography (see METHODS, results not shown) included numerous fluorene-containing products, one group eluting in the area of the starting material at about 30% acetonitrile. These may include hydroxylated products. Another group of products eluted at about 50% acetonitrile. There was no 8-hydroxy-deoxyguanosine or 8-amino-deoxyguanosine and only a trace of deoxyguanosine at the beginning of the gradient. Yet this area contained over 90% of the deoxyribose according to the diphenylamine assay of Burton (24).

We conclude from these results that the oxidation, which starts at the NH of deoxyguanosin-8-yl-AF, also destroys the guanosine chromophor. The

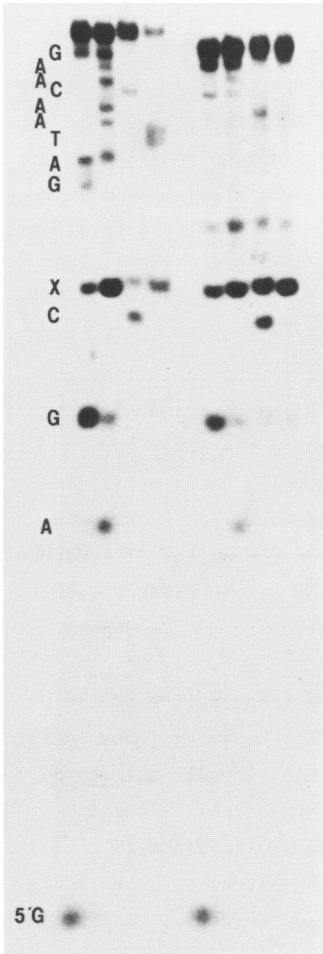

FIG. 5. Sequencing gel of 14-mers containing guanine-8-yl AF (left) and an oxidation product of guanine (right) in position X. Base-specific reactions are described under Methods and are from Left G, A>G, C and T.

resulting structure, perhaps a derivative of deoxyribosylallantoin, is stable enough to allow isolation of the full-size oligonucleotide but cleaves at the position of this oxidized structure on treatment with piperidine under the conditions of sequence analysis.

Isolation

Anion exchange chromatography, after prior desalting of the crude oligonucleotide, affords a rough size fraction as shown in Figure 4, left panel, and removes some nonnucleotidic material which elutes early. Complete purification is achieved by subsequent reverse phase chromatography. Figure 4, right panel, shows the elution diagram of the high molecular fraction from the preceding DEAE column. The lipophilic AF-moiety causes the 14-mer to

elute at 25% acetonitrile instead of 13%. The peak near 40 ml (separate chromatographic run) represents a 14-mer that has lost the AF-moiety as a result of air oxidation at high pH and the remaining structure is the guanine oxidation product discussed above. The material in the peak near 140 ml gives a single band on a sizing gel and was used for sequence analysis.

Sequence Analysis

The sequencing gel in Figure 5 shows the effect of the bulky substituent on the electrophoretic mobility. 14-mers containing guanin-8-yl-AF (left) and a guanine oxidation product in place of guanine (X) are sequenced side by side. Base cleavage patterns are normal up to the modified bases which both react with all four reagents used. During base-specific chemical cleavage, the base that underwent the base-specific reaction is lost and therefore the band corresponding to X has a normal spacing relative to the lower and smaller fragments. The next band higher and all following bands still contain the base X and in the left sequencing ladder show the greatly reduced electrophoretic mobility caused by the AF-moiety (electrophoretic differences are enhanced in this 24% gel). The comparable spacing in the right sequencing ladder is more normal, reflecting the smaller difference between guanine and the guanine oxidation product.

Other effects of the AF-moiety include a uniform exaggeration of the A+G lane and a doubling of the T band. The latter is a known artifact in the permanganate reaction and the C+T hydrazine reaction (result not shown) shows only a single band corresponding to the upper of the twin T bands. Some faint bands in the left sequencing ladder correspond to bands in the right and may be caused by the oxidative side reaction discussed above since no mercaptoethanol was added to the piperidine cleavage reaction. In the right sequencing ladder the effect of the guanine oxidation product is more severe, rendering the upper part of the sequencing ladder unintelligible.

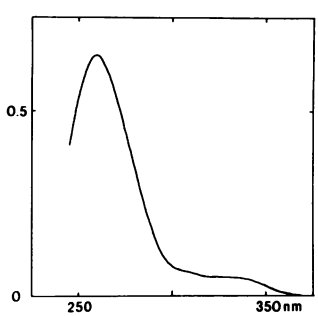

FIG. 6. Ultraviolet absorption spectrum of pure AF-containing 14-mer in 0.1 M TEA-acetate, pH 7.0, containing 25% acetonitrile.

This is mostly due to the instability of the oxidized base under conditions of piperidine cleavage.

Purity of the Oligonucleotide

The UV-spectrum of the purified tetradecamer containing the AF-moiety is shown in Figure 6. The ratio of absorbances A_{260}:A_{280}:A_{300} is 1:0.54:0.12 and is in good agreement with the values of 1:0.54:0.135 calculated according to Büchi et al. (16) and using a $\varepsilon_{300} = 25 \times 10^3$ for the deoxyguanosin-8-yl-AF adduct. For the biological experiments planned, an assessment of the purity of the modified oligonucleotide is important. We feel the good separation of the fluorene containing oligonucleotide from oligonucleotides that have lost the fluorene moiety allows isolation of essentially pure modified oligonucleotide after repeated reverse phase chromatography. The modified oligonucleotide appears stable once isolated. At pH ~6 no loss of fluorene can be noticed.

In summary, we have synthesized an oligonucleotide containing the major DNA adduct formed by the carcinogen acetyl aminofluorene. Such site-specifically modified oligonucleotides will allow us to ask new questions on the role of bulky base adducts in mutagenesis, DNA repair and gene regulation.

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